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## Some problems and errors in cytogenetic biodosimetry

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### ABSTRACT

Human radiosensitivity is a quantitative trait that is generally subject to binomial distribution. Individual radiosensitivity, however, may deviate significantly from the mean (by 2–3 standard deviations). Thus, the same dose of radiation may result in different levels of genotoxic damage (commonly measured as chromosome aberration rates) in different individuals. There is significant genetic component in individual radiosensitivity. It is related to carriership of variant alleles of various single-nucleotide polymorphisms (most of these in genes coding for proteins functioning in DNA damage identification and repair); carriership of a different number of alleles producing cumulative effects; amplification of gene copies coding for proteins responsible for radioresistance, mobile genetic elements and others. Among the other factors influencing individual radioresistance are: the radioadaptive response; the bystander effect; the levels of endogenous substances with radioprotective and antimutagenic properties and environmental factors such as lifestyle and diet, physical activity, psychoemotional state, hormonal state, certain drugs, infections and others. These factors may have radioprotective or sensibilizing effects. Apparently, there are too many factors that may significantly modulate the biological effects of ionizing radiation. Thus, conventional methodologies for biodosimetry (specifically, cytogenetic methods) may produce significant errors if personal traits that may affect radioresistance are not accounted for.

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factors

### Abbreviations

ERCC1	–	excision repair, complementing defective
HPV	–	human papilloma virus
IR	–	ionizing radiation
MLH	–	homologue of MutL of <i>E. coli</i>
RRM1	–	ribonucleotide reductase
SCID	–	severe combined immunodeficiency
SD	–	standard deviation from the mean
SOD	–	superoxide dismutase
XPC	–	Xeroderma pigmentosum complementation group C
XPD	–	Xeroderma pigmentosum complementation group D
XPG	–	Xeroderma pigmentosum complementation group G
XRCC	–	X-ray repair complementing defective
UTR	–	untranslated region

### Introduction

The correlation between the received dose of ionizing radiation (IR) and its biological effects (increases in the levels of intracellular free radical species, increased level

of genomic instability, increased mutation rate, induction of apoptosis, increased risk for neoplastic transformation) is not always straightforward. Several cases of naturally occurring extreme radioresistance in humans have been reported, usually in case reports of survivors of industrial accidents [1,2]. Thus, simple calculation of the received dose may not be sufficient when assessing the effects of exposure to IR. Biological dosimetry is based on assessment of genotoxic damage using specific biomarkers and may provide a more reliable source of information about the effects of IR. The first validated methodology for biological dosimetry (the dicentric assay, based on analysis of solid stained dicentric chromosomes in lymphocytes from peripheral blood) was introduced in the mid-1960s. For decades, the dicentric assay was the only method of biological dosimetry available, and in our day, it is still the technique that is most frequently used. There may be, nevertheless, a discrepancy between the assessment of the effects of irradiation obtained *in vitro* (using whole blood or cultured lymphocytes) and *in vivo*. Basically, the dicentric assay produces results that correlate well with the irradiation dose when carried out in cultured cells, but may not be reliable

when assessing the effects of IR *in vivo*, as there are many factors that affect individual radiosensitivity, endogenous as well as exogenous, in living organisms. The major factors determining individual radiosensitivity that may be source of biodosimetric errors are reviewed below.

### Binomial distribution of radiosensitivity vs. individual radiosensitivity

Radiosensitivity is a quantitative trait. Its distribution within the population is characterized by a 'bell-like curve'. Thus, approximately 50% of the population exhibits an 'average' radiosensitivity ( $x \pm 0.67$  standard deviations (SDs)). The majority (95%) of individuals within the population exhibit radiosensitivity of  $x \pm 1.96$  SDs. Only approximately 5% of the population falls within the range of  $x \pm 1.96$  SDs to  $x \pm 3$  SDs. The latter group may be sub-divided further into the extremely radiosensitive (2.5%) and the extremely radioresistant fraction (the remaining 2.5%).

The proportion of people that exhibit sensitivity to irradiation increases with increase of the received dose, although the correlation is not directly proportional. There is a steep increase in the proportion of radiosensitive individuals with increase of the dose, corresponding to the 'extremely sensitive' individuals and the 'average-sensitive' individuals, followed by a steep decline in the number of radiosensitive individuals, corresponding to the 'extremely radioresistant' fraction of the population. There has always been some contradiction about the existence of a 'threshold' dose of IR that may result in genotoxic effects (that is, whether a dose below the 'threshold dose' may not be associated with any genotoxic effects). The existence of an 'extremely radiosensitive' fraction, however, may invalidate the theory of the 'threshold dose', as there is always a small proportion of individuals in a population that may suffer lasting effects from a dose that causes no harmful effects in the majority of individuals. Indeed, if the study cohorts are too small or the assays used to assess genotoxic effects exhibit low sensitivity tests, 'extreme radiosensitivity' may not be registered, which would manifest as an apparent dose 'threshold' followed by a steep increase in radiosensitivity.

Apparently, individual radiosensitivity may differ significantly from the 'average' radiosensitivity within a population, and, respectively, the same received dose may result in dissimilar genotoxic effects.

There is significant genetic component in individual radiosensitivity. It is related to carriership of variant alleles of various single-nucleotide polymorphisms (most of these in genes coding for proteins functioning in DNA

damage identification and repair), carriership of a different number of alleles producing cumulative effects, amplification of gene copies coding for proteins responsible for radioresistance, mobile genetic elements and others. Among the other factors influencing individual radioresistance are: the levels of endogenous substances with radioprotective and antimutagenic properties; the adaptive response and the bystander effect; and environmental factors such as lifestyle and diet, physical activity, psychoemotional state, hormonal status, certain drugs (e.g. cytostatic medication), infections and others. These factors may have radioprotective or sensibilizing effects.

### Individual capacity for DNA repair

The efficiency of repair of DNA damage inflicted by IR may be quite dissimilar in different individuals. Exquisite sensitivity to IR and radiomimetic drugs (bleomycin, neocarzinostatin and others) is part of the clinical picture in inherited disorders of DNA damage-associated response and/or repair such as ataxia-telangiectasia, Nijmegen breakage syndrome and various severe combined immunodeficiency (SCID)/radiosensitivity syndromes [3–6]. Even among the clinically healthy individuals, however, the response to DNA damage may greatly vary. It is currently believed that individual radioresistance is determined by a variety of factors, including specificities in the genetic background, specificities in the physiology of the cells and tissues that received the irradiation, and environmental factors [7,8].

Carriership of a common polymorphism in the *XPC* gene coding for a major protein of DNA damage recognition and repair has been associated with increased sensitivity to bleomycin [9]. This and other polymorphisms in genes of DNA repair (*XPB* Lys751Gln, *XPG* Asp1104His, *XRCC1* Arg399Gln and *XRCC3* Thr241 Met) were associated with increased levels of strand breaks and chromosomal aberrations in healthy human volunteers [10].

It is well known that tissues with rapid turnover such as bone marrow and epithelia are significantly more vulnerable to the effects of IR than tissues containing long-lived cells that are rarely (if ever) replaced, such as neuronal tissue. The latter is believed to be related to the principle of parsimony of DNA repair mechanisms typical of the DNA repair profile in certain species ('the rodent repairadox') and in certain types of cells (adult neurons, memory B-cells) that ensures that only transcribed DNA is repaired [11–13]. Indeed, cells that are not expected to divide may not be subjected to the genome integrity checks that are routinely carried out in dividing cells and are, therefore, not likely to be assessed as too damaged

to keep [14,15]. This mechanism seems to ensure neuronal lifespan roughly comparable to the lifespan of the organism, although some authors speculate that increased attrition of adult neurons secondary to oxidative damage and poor supply of replacement neurons from the neuronal stem cell niche may constitute an important component of the pathogenetic mechanism of late-onset neurodegenerative disease [16–18]. In any case, assessment of IR-inflicted damage may show very different results when carried out in radiosensitive and radioresistant types of cells and tissues.

Modern anticancer therapy (including radiotherapy) is largely based on suppression of proliferation of cancer cells by inflicting enough damage in their DNA so as to activate the mechanisms that ensure that cells with compromised genome integrity are not replicating their DNA. More aggressive therapies are therefore more likely to produce the desired effect of staving off tumour growth. Nevertheless, agents and/or treatments that produce rapid and severe suppression of cell division are inevitably associated with increased rates of adverse effects (anaemia, agranulocytopenia, mucositis, epilation, etc.). Carriership of polymorphisms in genes coding for proteins of DNA damage-associated response and/or DNA repair may be associated with differential response to radiotherapy in terms of survival and/or rates of adverse effects [19,20]. Low levels of mRNA of proteins acting in replicative synthesis of DNA (*ERCC1*, *RRM1*) and heterozygous carriership of the single-nucleotide polymorphism *ERCC1* T19007C may be associated with increased chances for tumour regression/increased survival in patients with certain types of solid tumours treated with radiotherapy alone or as part of a combined chemotherapy/radiotherapy regimen [21–24].

The locus containing the *TP53* gene is altered (mutated or altogether deleted) in about half of human cancers [25]. Modification of the *TP53* gene in tumour cells is usually associated with poorer prognosis for the patient [25,26]. *P53* is activated in response to acute DNA damage caused by radiation [27] and at least in some tumours, loss of the *TP53* locus may be associated with increased risk of radioresistance [28]. In HPV-related human tumours such as cervical carcinoma and head and neck cancers, the presence of viral DNA in the tumour tissue may be associated with better prognosis for the patients (reviewed in [29]). Recently, the presence of human papilloma virus DNA in tumour samples has been reported to be associated with increased radiosensitivity as well [30].

Carriership of the *XRCC1* Arg399Gln, *XRCC3* Thr241Met and *SOD2* codon 16 polymorphisms may be associated with increased risk of subcutaneous fibrosis after radiotherapy [31,32]. The presence of *XRCC1*

399Gln and *APE1* 148Glu polymorphisms in breast cancer patients was associated with reduced risk for moist desquamation of irradiated normal skin in patients with breast cancer treated with radiotherapy [33]. The very common *TP53* Pro72Arg polymorphism was found to be associated with development of atypical vascular lesions at the sites of radiotherapy for breast carcinoma [34]. The polymorphisms *LIG4* Asp568Asp, *XPD* Asp711Asp, the 5'-UTR A > G polymorphism at nt4541 in the *XRCC3* gene and *MLH1* Val219Ile are associated with late rectal and/or bladder toxicity in patients treated with radiotherapy for prostate cancer [35]. *ATM* protein is one of the key radiation sensors in the cell [36,37] and several polymorphisms in the *ATM* gene were found to be associated with increased risk for severe radiation pneumonitis, a common complication in patients with lung cancer treated with radiotherapy [38].

The risk of anticancer therapy-related toxicity conferred by virtually all of the genetic factors listed above may be modulated by other factors, including environmental factors (smoking, obesity, etc.). Obesity is a major risk factor for acute toxicity in virtually all types of anticancer treatments [39–41]. This adverse effect is most likely related to the higher dose of the genotoxic agent that is required to achieve the desired effect in larger patients. History of smoking and continuing to smoke after receiving the diagnosis of cancer may result in up-regulation of the mechanisms of DNA repair, resulting in more efficient elimination of the DNA damaging agents of genotoxic therapy. Studies show that the response to radiotherapy was poorer in smoking patients with head and neck cancers than in non-smokers [42].

Apparently, the genetic background plays a major role in the constitution of individual radiosensitivity, or, as the famous radiobiologists Mothersill and Seymour propose: 'Clearly, genetic predisposition is crucial and may even be more important than dose' [43]. This, nevertheless, is valid only within certain limits, as it is presently believed that there are natural mechanisms that prevent both 'ultimate resistance to mutation' and 'ultimate propensity for mutation' in order to keep the evolution going [44,45].

Analysis of DNA polymorphisms in genes coding for products responsible for identification and repair of DNA damage inflicted by IR may be complemented by methodology that assesses the outward expression of the overall capacity for repair of damage (phenotypic analysis). There are several methods that may be used to monitor the overall capacity for repair of induced damage of a specific type and/or the genomic stability in selected sequences [46–48]. The methodologies described in [46] and [48] have been developed to assess levels of ultraviolet (UV)-induced damage, but it may be

expected that they could be easily adapted for assessment of damage inflicted by IR.

### Radioadaptive response

The phenomenon of radioadaptive response is another major factor that may be responsible for the discrepancies between the anticipated effects of the received dose and the biological effects *in vivo*. By definition, the adaptive response is a specific reaction that occurs on the level of whole cells, tissues or organisms in response to stress. The adaptive response is dose-dependent, with the adapting (priming) dose being significantly smaller (sometimes by several orders of magnitude) than the dose that causes acute damage. Typically, the priming dose does not cause any detectable damage. However, when a larger dose (usually associated with acute damage) is received following a priming dose, a significant reduction in the rate of occurrence of the associated harmful effects may be noted. Usually, administration of a priming irradiation dose reduces the effects of an acute radiation dose by a factor of 2 [49]. Apparently, the radioadaptive response can modify biodosimetry results.

Radioadaptive response may be elicited not only by a smaller priming dose of irradiation, but also by agents other than IR, such as thermal shock, chemical mutagens and others, the majority of which could not be controlled under normal conditions (stress, vitamin and antioxidant intake, etc.). What is more, the protective effect of a priming dose may be observed not only when the priming and the damaging agent are of the same nature (e.g. a smaller dose of radiation followed by a larger dose), but also when the priming and the damaging factor are different (e.g. a small dose of UV irradiation may protect from the harmful effects of a dose of IR that is usually associated with acute damage). The protective effects of the radioadaptive response may be observed on many levels, using biomarkers for genotoxic damage such as rates of occurrence of chromosomal aberrations, mutation rates, the micronucleus assay, etc. For damage caused by IR, it has been observed that the time interval between the priming dose and the damaging dose that is associated with the strongest radioadaptive response is 5–6 hours [49]. Reducing the time interval between exposures results in decrease of the radioadaptive response. Increasing the time interval between subsequent doses up to 10 hours does not result in decrease of the radioadaptive response [49].

The adaptive response may be observed with some damaging agents but not with others. In the late 1970s and early 1980s, significant variation was observed in the repair responses of cultured human lymphocytes from healthy volunteers to DNA damage induced by

chemical agents [50,51]. This, however, may not pertain to all types of genotoxic damage, as there are reports in the specialized literature that there was virtually no measurable radioadaptive response in lymphocytes from unrelated healthy volunteers [49,52]. In any case, the results of assessments of genotoxic damage may be quite different in cases when a priming dose has been administered and in cells, tissues and organisms that are naive to IR.

The radioadaptive response may be modulated by different agents. It was previously shown that the pigment melanin reduced the mutation rates by a factor of 2 and contributed to the adaptive response, resulting in reduction of the rate of chromosomal aberrations by a factor of 4 [53,54]. In a previous study, we revealed that certain mutagens such as the herbicide Senkor prevented the adaptive response by inhibiting repair processes but antioxidants such as  $\alpha$ -tocopherol can alleviate this effect [55].  $\alpha$ -Tocopherol (commonly known as vitamin E) is a fat-soluble vitamin compound naturally occurring in mammalian cell membranes, and it is a potent free-radical scavenger believed to play a major role in the natural defence against lipid peroxidation in cell membranes [56]. The radioprotective effect of tocopherol in the small intestine (notoriously known for its rapid cell turnover and, therefore, for its being among the main targets for radiation toxicity) has been directly demonstrated by Franza et al. in mice [57]. Other authors [58] reported that oral administration of tocopherol prior to gamma-irradiation in male mice had a radioprotective effect on spermatogenesis. Considering the famous 'rodent repairadox' phenomenon, it is unclear whether these findings may apply to cells from other species, and especially human cells. Laurent et al. [59], however, showed that the combination of  $\alpha$ -tocopherol and the peripheral vasodilator pentoxifylline in skin fibroblasts was highly efficient in reducing delayed radiation-induced damage. It was found that the protective effect of  $\alpha$ -tocopherol may conceal a radioadaptive response in murine bone marrow cells [55].

### Bystander effect

Like all types of electromagnetic radiation, IR travels in a straight line until absorbed or deflected. Thus, it is possible that a narrow beam of IR may irradiate only an isolated portion of the body, although there is always a degree scattering as IR traverses living tissue. The latter allows for fine shaping of external radiotherapy beams and may be among the causes for survival of individuals that have received high doses of IR (if we could remember the infamous case of Anatoly Bugorski, a scientist from the former Soviet Union who survived after



receiving an estimated dose of 200 Gy to the head, albeit in a narrow beam). Nevertheless, it has been well documented that when a cell population (*in vitro* or *in vivo*) is exposed to IR, the effects of the irradiation may be observed in a larger proportion of cells than may be assumed to have been directly irradiated. This phenomenon (bystander effect) is usually explained by cell–cell signalling between irradiated cells and closely located non-irradiated cells. Specifically, the bystander effect has been attributed to communication between irradiated and unirradiated cells by direct contact between neighbouring cells; signalling via specific substances in the nutrient medium of irradiated cells that influenced unirradiated cells and immunity-mediated responses [43,60,61]. Microarray studies revealed that the profile of gene expression in irradiated cells was different from that in unirradiated cells which exhibited evidence of bystander effect, with only part of the genes whose expression has been modulated in the irradiated cells being altered in unirradiated cells receiving media from irradiated cells [62]. Moreover, the bystander effect could reportedly be elicited in unirradiated cells using cells that have received irradiation decades ago [63]. Bystander effect is believed to be at least partially responsible for the risk of occurrence of secondary leukaemia in cancer survivors that have been treated with radio- or chemotherapy for the primary tumour [64,65]. It is possible that bystander effect may account for at least some of the errors in biodosimetry by conventional cytogenetics, as the effects of irradiation such as chromosome instability may be manifested in bystander cells that have not received irradiation but may, similarly to irradiated cells, be prone to increased rates of cell death and/or carcinogenic transformation.

### Environmental factors

Individual radiosensitivity may depend (sometimes significantly) on environmental factors, such as lifestyle and diet, physical activity, psychoemotional state, hormonal status, use of certain drugs, infections, etc. Those are unspecific factors that are very common and are prone to periodic and/or sudden change; therefore, it is difficult to take these into assumption when assessing the reliability of biodosimetry results. There are also many specific environmental agents (e.g. industrial chemicals) that are quite common and may seriously interfere with biodosimetric assessments, as they are capable of modulating the genotoxic effects of IR. The effects of these agents (alone or in combination with other agents) may not be studied well enough to predict the effects they may have on biodosimetry results. Residual amounts of industrial chemicals that are known (to affect individual

radioresistance (e.g. fertilizers, herbicides, etc.) may easily leak into human food and water (e.g. via contamination of groundwater [66]) and may modulate the cellular response to genotoxic insults. The delayed effects may be difficult to predict, as they occur as a result of a complex interplay of the genetic background, the lifestyle factors and the additional environmental challenge provided by the genotoxic agent/s. At present, the data about the delayed genetic effects of combinations of different genotoxic agents is quite limited, although there have been studies of the effects of combined genotoxic challenge on mammalian cells (physical and chemical agents together, e.g. IR and genotoxic chemicals) [53,67,68] and of combinations of genotoxic chemicals – commonly used fertilizers (sodium nitrite, sodium nitrate) and the herbicide Sencor® (metribuzin) in mice and human cells. Specifically, it was found that sodium nitrite and sodium nitrate administered *per os* in amounts that were not associated with an increase in the overall mutation rate, significantly sensitized irradiated mice to the genotoxic effects of IR (expressed as 2–4-fold increases in the rates of cytogenetically detectable chromosomal aberrations) [68]. There have also been studies of the potential effect of Sencor® on the radiosensitivity of irradiated mice [67]. Unlike sodium nitrite and sodium nitrate, which had no detectable mutagenic effects in low concentrations, intraperitoneal or oral administration of Sencor® in unirradiated mice and adding of Sencor® to the nutrient medium of unirradiated human cultured cells resulted in an increase in the rate of occurrence of chromosomal translocations. Application of Sencor®, however, resulted in reduction of the levels of chromosomal aberrations in murine germ cells [67,68]. The effect was more pronounced with chronic administration of the genotoxic combination Sencor/irradiation than in acute settings. This effect may be explained by stimulation of the programmed cell death pathway in cells that have sustained too much damage. Generally, the mode of action of Sencor® in plant cells is based on inhibition of the electron flow between the primary and secondary electron acceptors of photosystem II [69]. In mammalian cells, a similar effect may disrupt the electron transport chain of oxidative phosphorylation, resulting in increase of the reactive oxygen species and subsequent oxidative damage (oxidized bases in DNA, double-strand breaks). This may explain the mutagenic properties of Sencor® in mammalian cells. IR causes more than one type of damage in DNA, including oxidative damage. The latter is a potent inductor of the DNA repair response pathway, but also of programmed cell death [70]. Thus, it is possible that the concomitant administration of both genotoxic agents may result in rapid induction of apoptosis, leaving very little viable

cells with detectable translocations. Similar results have been obtained with *Drosophila* [53].

### Radioprotective properties of melanin

Melanin is a skin pigment that plays a variety of roles, including protection of the germinative layer of the skin from the harmful effects of UV irradiation [71]. Melanin is produced in specialized cells (melanocytes) in the skin and its appendages (the basal layer of the skin and the hair follicle), in the eye (choroidal melanocytes and retinal pigment epithelial cells), the inner ear, the brain meninges and in other tissues and organs, such as the liver, the heart, the bone and the adipose tissue. Principally, the amount and type of melanin produced in the mammalian skin and eye are genetically determined, but this may be modulated by a variety of factors, including age, hormonal status and exposure to UV light. Melanin is an efficient converter of electromagnetic energy. It participates readily in processes of electron transfer and is an efficient free-radical scavenger [71–73]. Beverages such as tea, coffee and cocoa and foodstuffs such as grapes, bananas and edible fungi contain melanin-like pigments [74,75]. There have been reports that melanin could be among the causes for the phenomenon of increased survival of fungi growing on nuclear test sites after receiving multiple lethal radiation doses (after soil irradiation with 6400 Gy) [76]. It has been shown that the populations of plants with intensive pigmentation increased in areas contaminated with  $^{90}\text{Sr}$  [77]. The phenomenon of hyperpigmentation (on selected sites, accompanied by fur greying) was also described in mice undergoing daily gamma-irradiation [78]. Brunst [79] reported that irradiation of young axolotls with increasing doses of IR stimulated the production of melanin in the liver, the brain and the eyes, which he viewed as a potential protective mechanism.

Studies on the antimutagenic properties of melanin are still sparse in the specialized literature. Previous reports show that melanin may significantly decrease the rates of occurrence of different types of IR-induced mutations in germinative cells of model animals (*Drosophila*, mice) and in cultured human keratinocytes [80–83]. Melanin has been shown to protect against the harmful effects of IR not only in acute settings, but also after chronic exposure [84]. There have been a number of studies dedicated to the potential uses of melanin as an agent increasing biological radioresistance. Berdishev [85] found increased survival and longer life expectancy of irradiated white mice that have received intraperitoneal injections of melanin prior to irradiation with 8 Gy, a dose that normally results in death of mice after 6–14 days. Nevertheless, as rodents have a unique DNA

repair profile, the results obtained with mice may not be directly applicable to other species, including humans.

It is possible that increased concentrations of melanin in the skin and the viscera may alleviate the effects of exposure to IR. Melanin exerts its protective action at the initial stage of irradiation, absorbing and converting high-energy electromagnetic radiation and reducing the levels of free radical species, thereby preventing DNA damage [71,86]. Thus, individual characteristics such as melanin content may affect individualized biodosimetric measurements.

### Conclusions

Radiosensitivity is subject to individual variation, i.e. the responses of different individuals to the same absorbed dose of radiation may be quite different. This natural variation is related primarily to differences in the genetic background due to carriership of variant alleles of genes coding for products functioning in DNA repair but may be modulated by other factors as well (radioadaptive response induced by previous exposure to low-dose electromagnetic radiation and/or exposure to specific chemical agents, bystander effect, etc.). Environmental factors may also play a role. Lifestyle changes, changes in hormonal status, acute or chronic stress and infections may also affect individual radiosensitivity. Popularly used beverages and foodstuffs may contain substances with antioxidant properties (e.g. tocopherol, melanin and others) and/or residual amounts of agents (fertilizers, herbicides, etc.) that may modulate response to IR. The contribution of these largely uncontrolled factors may be responsible for significant errors of biodosimetric evaluations. Moreover, the cells that are most commonly collected for the purposes of individualized analysis (peripheral lymphocytes) appear not to reflect correctly the levels of radiation damage in other tissues and organs and the organism as a whole. Future studies may be needed in order to elucidate the relationship between received dose and individual reaction to radiation damage.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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